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Claim 60
Cytosine deaminase

TECHNICAL ADVANCE

Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene

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Summary

The inability of many higher eukaryotes to convert 5-fluorocytosine to cytotoxic 5-fluorouracil presents the possibility of using the bacterial cytosine deaminase *codA* gene for negative selection. In transformed plant callus, expression of *codA* results in cell death on 5-fluorocytosine. In transgenic tobacco and *Lotus japonicus* plants the substrate-dependent negative marker segregates as a single dominant gene, and on 5-fluorocytosine CodA$^+$ seedlings stop growing at the early seedling stage. Positive selection of CodA$^+$ tobacco on the pyrimidine biosynthetic inhibitor *N*-(phosphonacetyl)-L-aspartate was obtained, by pyrimidine salvage from external cytosine. Activity of cytosine deaminase was determined by conversion of labelled cytosine to uracil followed by separation in thin layer chromatography. The *codA* marker therefore provides substrate-dependent negative and positive selection, together with cytosine deaminase reporter activity.

Introduction

The use of positive genetic markers conferring resistance towards antibiotics or herbicides is crucial for the selection of transformed plant cells and a prerequisite for the production of transgenic plants. To establish refined cellular and genetic techniques, as for example gene targeting by homologous recombination, negative marker genes counter-selecting cells expressing the gene are also essential. Developmentally programmed cell death following expression of a cell autonomous negative marker could provide new possibilities for the analysis of cell lineages and cell differentiation processes. At present two negative plant markers have been described. The *Agrobacterium tms2* gene encoding indole-3-acetamide hydrolase converts supplied indole-3-acetamide substrate into toxic levels of the auxin indole-3-acetic acid (Depicker *et al.*, 1988). Recently, a constitutively expressed nitrate reductase gene was used as a negative selection marker on nitrate-deficient growth medium. In tobacco the endogenous nitrate reductase was not induced under these conditions and negative selection was possible on chlorate (Nussaume *et al.*, 1991). A novel substrate-dependent negative selection marker based on the *Escherichia coli* cytosine deaminase *codA* gene is presented here. When *E. coli* utilizes cytosine through the pyrimidine salvage pathway, cytosine permease and cytosine deaminase mediate the uptake and conversion of cytosine into uracil (Danielsen *et al.*, 1992) (Figure 1). Cytosine deaminase also deaminates the innocuous 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a precursor of 5-fluoro-dUMP which irreversibly inhibits thymidylate synthase activity. Consequently the cells are deprived of dTTP for DNA synthesis (Brockman and Anderson, 1963). In plants and mammals enzymatic conversion of cytosine to uracil is absent and cytosine appears biochemically inert (Bendich *et al.*, 1949; Ross, 1965). In mammals, low toxicity of administered 5-FC is reflected in rapid and almost quantitative excretion (Koechlin *et al.*, 1966).

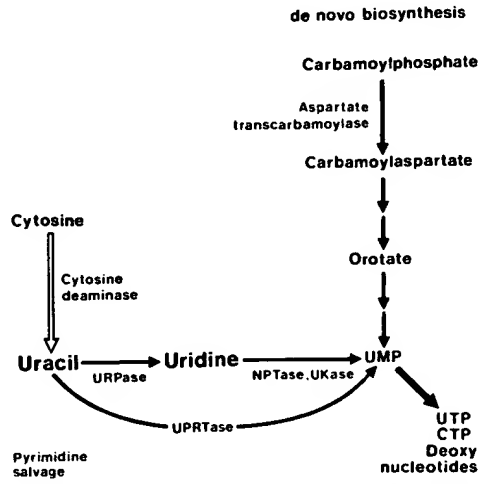

Figure 1. Schematic outline of the plant pathway for UMP biosynthesis, and pyrimidine salvage (Kanamori *et al.*, 1980).
The first committed step of *de novo* synthesis mediated by aspartate transcarbamoylase is at the top; pyrimidine salvage from uracil is left to right. Cytosine deaminase mediated conversion of cytosine to uracil in *codA* transgenic plants is indicated by an open arrow. URPase, uridine phosphorylase; UKase, uridine kinase; NPTase, nucleoside phosphotransferase; UPRTase, uracil phosphoribosyltransferase.

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Negative selection of mammalian culture cells on 5-FC was, however, observed after transfection with a marker gene expressing bacterial cytosine deaminase (Mullen *et al.*, 1992). This paper describes the use of cytosine deaminase as a dual selection marker and reporter gene in transgenic plants. It is shown that 5-FC does not affect the growth of wild-type plants, while transgenic plants expressing cytosine deaminase are negatively selected on 5-FC. The biochemical mechanisms behind the dual selections are discussed.

Results

Construction of the selectable marker

The cytosine deaminase coding sequence introduced into tobacco and the diploid legume *Lotus japonicus* was obtained from the plasmid pSD112 carrying the *codBA* operon (Danielsen *et al.*, 1992). Using PCR techniques the *E. coli* GTG translational start codon was changed to ATG before *codA* was fused to the nopaline synthase 3′ region and either the cauliflower mosaic virus 35S promoter (pNE7) or the 2′ gene promoter (pNE5) (Figure 2). Transgenic plants of *L. japonicus* (pNE7) and tobacco (pNE5) were obtained using described *Agrobacterium* procedures (Handberg and Stougaard, 1992; Horsch *et al.*, 1985). To confirm the presence of intact cytosine deaminase constructs in transgenic *Lotus* and tobacco plants, and to probe for cross-hybridization, genomic DNA was digested with *Cla*I (Figure 2) and hybridized to the *codA* coding sequence (Figure 3a). Transcription of the *codA* marker gene was demonstrated by hybridization of total RNA from leaves with the *codA* coding sequence. Transcript hybridization signals corresponding to the expected approximately 1400 nucleotides were detected in *codA* plants (Figure 3b). No cross-hybridizing RNA species were detected in *Lotus* and tobacco control plants, demonstrating the potential use of *codA* as a reporter gene for measuring transcript levels.

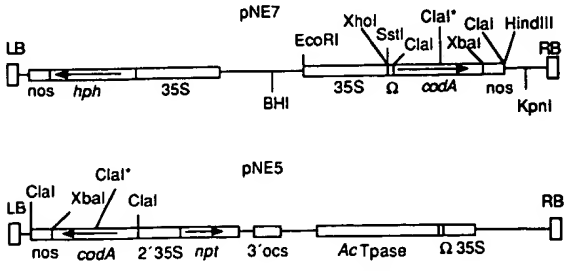

Figure 2. Schematic representation of the *codA* gene constructs used for expression of the bacterial cytosine deaminase in plants.
In the pNE7 plasmid, the 35S*codA* 3′*nos* gene was cloned into the binary vector pLX412 (Landsmann *et al.*, 1988). The 2′*codA* 3′*nos* gene of pNE5 was constructed by inserting a *codA* 3′*nos* *Cla*I fragment downstream of the 2′ promoter of the pSLJ1111 plasmid (Scofield *et al.*, 1992), replacing the GUS 3′*nos* fragment. **Cla*I site Dam methylated. Not to scale.

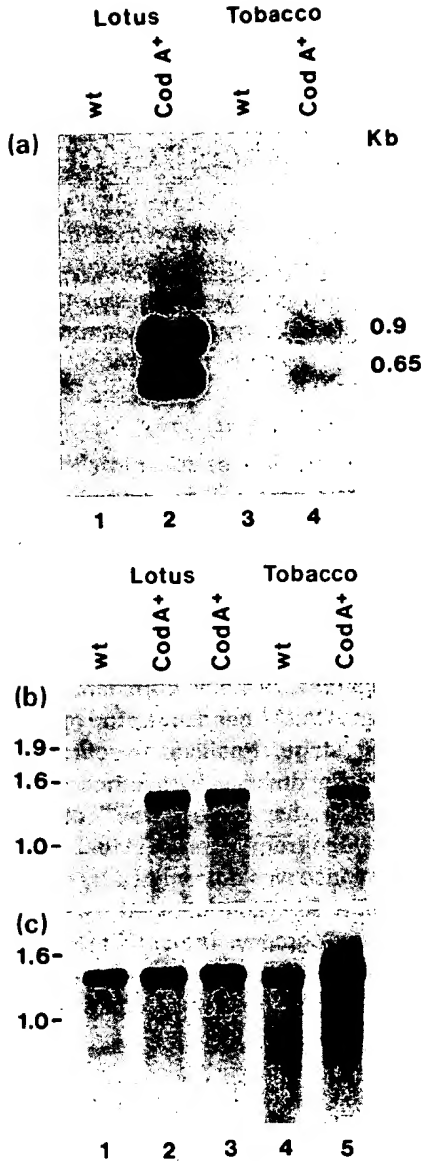

Figure 3. Southern and Northern analysis of transgenic *codA* plants.
(a) DNA extracted from *Lotus japonicus* and tobacco plants digested with *Cla*I. The 0.9 kb and 0.65 kb fragments encompassing the *codA* coding sequence and the 3′*nos* region (Figure 2), are detected in the transgenic plants (lanes 2 and 4) after hybridization with the *codA* coding region. No cross-hybridization in control plants (lanes 1 and 3). (b) Three micrograms of total RNA from *Lotus* and 12 μg total RNA from tobacco plants were hybridized with the *codA* coding sequence, or, (c) a cDNA probe for the constitutively expressed ubiquitin genes (Wiborg *et al.*, 1985). Transcripts of approximately 1400 nucleotides are detected by the *codA* probe in the transgenic *Lotus* and tobacco plants (lanes 2,3 and 5). Control plants represented by lanes 1 and 4.

Negative selection in calli

In callus medium the cytotoxic 5-FU prevented growth and caused death of *L. japonicus* calli, at levels of 50–100 μg ml^{-1} (Figure 4c). Concentrations of 5-FC up to 2 mg ml^{-1} had no observable effects. The negative selection was therefore tested under tissue culture conditions by

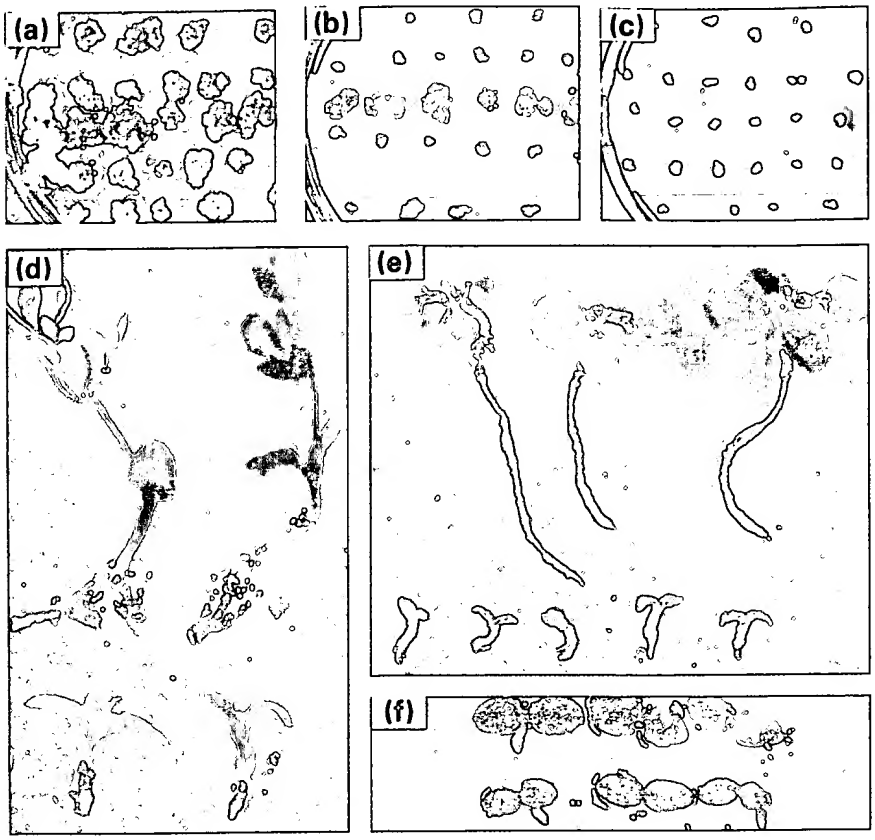

Figure 4. Negative and positive selection using 5-fluorocytosine and *N*-(phosphonacetyl)-L-aspartate.
Slices of *Lotus japonicus* calli carrying 35S*codA* 3′*nos* (two top and two bottom rows) and calli transformed with the pLX412 vector (middle row), were grown for 2 weeks. (a) Callus medium. (b) Callus medium containing 1 mg ml^{-1} of 5-fluorocytosine. (c) Cytotoxic effect of 100 $\mu g\ ml^{-1}$ 5-fluorouracil. (d,e) Phenotypes of *Lotus japonicus* and tobacco F_1 plants germinated and grown on 250 $\mu g\ ml^{-1}$ of 5-FC, $CodA^0$ (top) and $CodA^+$ (bottom). (f) Phenotypes of $CodA^+$ (top) and $CodA^0$ (bottom) F_1 tobacco plants germinated on 400 $\mu g\ ml^{-1}$ PALA plus 50 $\mu g\ ml^{-1}$ cytosine.

moving *codA* transformed *Lotus* calli from callus medium (Handberg and Stougaard, 1992) to similar plates containing 5-FC at 500 $\mu g\ ml^{-1}$ or 1 mg ml^{-1} (Figure 4b). To increase 5-FC uptake small pieces of calli were transferred. Transgenic calli carrying the 35S*codA* 3′*nos* marker gene did not grow and were white in appearance. Control calli grew normally and were green. In an attempt to demonstrate that the effect of 5-FC depends directly on the cytosine deaminase activity, concentrations of cytosine up to 2 mg ml^{-1} and uracil up to 100 $\mu g\ ml^{-1}$ were added to plates containing 5-FC at 500 $\mu g\ ml^{-1}$. Unexpectedly, neither of the two pyrimidines counteracted the negative selection and uracil at concentrations higher than 50 $\mu g\ ml^{-1}$ even enhanced the effect of 5-FC. Differences in uptake, enzyme affinities, or metabolic control effects may explain these observations.

Negative selection in plants

To test the negative selection *in planta*, transgenic *Lotus* and tobacco plants were selfed and the F_1 seeds germinated on 250 $\mu g\ ml^{-1}$ 5-FC. Generally, germination of $CodA^+$ seeds was slow, root growth was inhibited and roots did not elongate into the agar. Shoot development was rudimentary and in *Lotus* only a single trifoliate leaf appeared (Figure 4d). Tobacco seedlings were small and white (Figure 4e). Growth of control plants or segregating transgenic $CodA^0$ plants were not observably affected by 5-FC. The viability of $CodA^+$ *Lotus* and tobacco plants and the possible rescue after negative selection were tested by transfer to non-selective plates containing orotate at 100 $\mu g\ ml^{-1}$. Resumption of growth occurred in around 40% of the plants of both species.

The stability of the negative selection marker was investigated by following the segregation of *codA* in the F_1 generation of four independent *Lotus* plants and three independent tobacco plants. Cytosine deaminase activity was determined both by direct biochemical assay in non-selected plants, and by the ratio of sensitive to unaffected plants on 5-FC plates (Table 1a). Irrespective of the method used, *codA* segregated in the 3:1 ratio expected for a single dominant marker. A minor deviation between the two ratios observed in some F_1 populations, e.g. the *Lotus* line of Table 1a, could be statistical variation or could arise from intrinsically poorly growing $CodA^0$ seedlings scored as $CodA^+$ on selective medium. Indeed, three seedlings without cytosine deaminase activity were found among 125 *Lotus* and tobacco seedlings scored as $CodA^+$. The negative selection appears quite tight as no cytosine deaminase activity was found in 208 *Lotus* and tobacco plants originally assigned the $CodA^0$ character. Lack of penetrance has so far not been detected.

Table 1. (a) Segregation of cytosine deaminase activity and 5-fluorocytosine sensitivity in the F_1 plants from one line of selfed *L. japonicus* (35S*codA*) and one line of selfed tobacco (2′*codA*). Plants for biochemical assay were grown without selection. (b) Segregation of PALA resistance and cytosine deaminase activity in F_1 plants from selfed tobacco transformed with pNE5. Cytosine deaminase activity was determined in plants rescued from selective plates

				Assay, CodA$^+$:CodA^0		
	No. of plants	Ratio	χ^2	No. of plants	Ratio	χ^2
(a) Growth test on 5-FC, sensitive: unaffected						
Lotus	105:27	3.9:1	1.45	57:21	2.7:1	0.15
Tobacco	165:58	2.9:1	0.13	55:20	2.8:1	0.11
(b) Growth test on PALA+cytosine, sensitive:resistant						
Tobacco	46:118	1:2.6	0.81	121:43	2.8:1	0.13

The predicted counteractive dilution effect of other pyrimidines was tested on tobacco. Seeds segregating *codA* were germinated on plates containing 150 μg ml^{-1} 5-FC and a molar excess of the following compounds: cytosine, uracil, thymine, cytidine, uridine, thymidine, cytosine and thymidine, uracil and thymidine. At the concentrations used, these compounds failed to reverse the negative effect of 5-FC; uracil again enhanced the effect. A combination of thymidine, deoxycytidine, and cytidine was more effective and reverted the shoot phenotype of CodA$^+$ tobacco plants to almost wild-type appearance. Apparently, other fluorinated pyrimidine compound(s), in addition to 5-fluoro-dUMP, interfere with pyrimidine metabolism.

Positive selection in tobacco

The potential use of cytosine deaminase as a dual purpose marker also providing substrate-dependent positive selection was investigated in tobacco grown on *N*-(phosphonacetyl)-L-aspartate (PALA), an inhibitor of the pyrimidine biosynthetic enzyme aspartate transcarbamoylase (Swyryd *et al.*, 1974; Figure 1). Growth and development of wild-type tobacco were retarded by 400 μg ml^{-1} PALA and positive selection for CodA$^+$ seedlings surviving on cytosine uptake through the pyrimidine salvage pathway, appeared feasible. On PALA cotyledons were yellow, and no roots developed. Addition of 50 μg ml^{-1} of cytosine to selective plates resulted in green cotyledons on transgenic CodA$^+$ tobacco seedlings, but the growth inhibition was not reversed (Figure 4f). The phenotypic difference between CodA$^+$ and CodA^0 seedlings was therefore not as clear as after negative selection, and the relationship between green cotyledons and cytosine deaminase activity was determined. Table 1b shows the 3:1 ratio between green and yellow cotyledons and compares directly with the cytosine deaminase activity determined in the same plants, rescued on medium containing orotate at 100 μg ml^{-1}. The enzymatic assays confirm the scored phenotypes with an error of less than 10%. Uridine or orotate reversed the effect of PALA in any genotype whereas uracil reversed the effect on wild-type tobacco to the same extent as cytosine in CodA$^+$ plants. Slow conversion of uracil into uridine or UMP could explain these differences.

The cytosine deaminase assay

A marker gene with an easy assay is useful in genetic segregation studies and allows direct correlation of gene expression with phenotype. For plant purposes, a modified (Andersen *et al.*, 1989) cytosine deaminase assay was therefore established. Crude extracts prepared from leaves homogenized with a 10-fold excess of extraction buffer were incubated with labelled cytosine. The uracil formed was subsequently separated from the substrate on cellulose TLC plates (Figure 5). Two substrate concentrations higher than the K_m of 0.53 mM (Andersen, 1979) were tested: (i) 0.9 mM 2-^{14}C cytosine; (ii) 0.9 mM 2-^{14}C cytosine plus 10 mM cytosine. For both reactions a linear relation between protein content and activity was observed up to a protein concentration of 2.1 μg μl^{-1} (crude 1:1). The detection limits for the assays were found at protein concentrations of 0.0042 μg μl^{-1} and 0.042 μg μl^{-1}, respectively (Figure 6a). For detection of low level activities the most sensitive condition was chosen as standard assay. To test the validity of this assay, activity was measured as a function of time (Figure 6b and c). Activity was linear for 1 h and the specific activity was 0.95 nmol uracil μg^{-1} protein h^{-1}. Approximately 15% activity was lost in 3 h. Therefore, the cytosine deaminase enzyme appears to be stable, allowing the employment of *codA* as a reporter gene for studies of gene expression in transgenic plants.

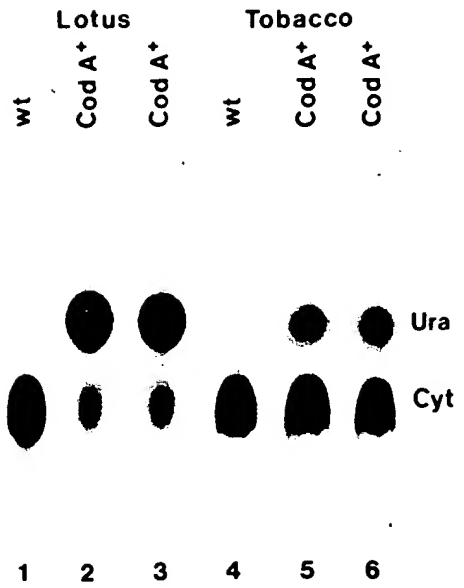

Figure 5. Determination of cytosine deaminase activity in plants. Conversion of the 2-^{14}C-cytosine (Cyt) substrate to 2-^{14}C-uracil (Ura) in extracts of CodA$^+$ *L. japonicus* and tobacco plants, lanes 2, 3, 5 and 6. Control plants, lanes 1 and 4.

Discussion

Expressed from the 35S promoter or the weaker 2′ promoter, cytosine deaminase provides a convenient negative selection in both *L. japonicus* and tobacco by converting the innocuous 5-FC to the cytotoxic 5-FU. The cytosine deaminase activity in the presence of 5-FC results in a distinct phenotype in both *in vitro* grown plant cells and plants. Judging by the absence of shoot development and root elongation on the sensitive plants, cell division in the root and shoot meristems appears to be inhibited or abolished. Rescue of around 40% of the growth-inhibited CodA$^+$ plants from 5-FC plates was nevertheless possible with both *Lotus* and tobacco. It will be interesting to test whether the negative effect is cell autonomous and *codA* therefore usable in programmed cell death experiments. This application will rely on 5-FC translocation throughout the plant and requires that fluorinated pyrimidines like 5-FU are not exported. The effect on the shoot meristem indicates good translocation, but the experiments reported can not exclude effects from exported fluorinated pyrimidines.

Some questions concerning the exact mechanism of the negative selection in plants are still unanswered. The inability of thymidine to reverse the 5-FC effect and the only partial reversion of the negative selection in tobacco observed with a combination of thymidine, deoxycytidine, and cytidine suggest that secondary effects of fluorinated pyrimidines might contribute to the negative selection in addition to inhibition of DNA synthesis. Inhibition of RNA

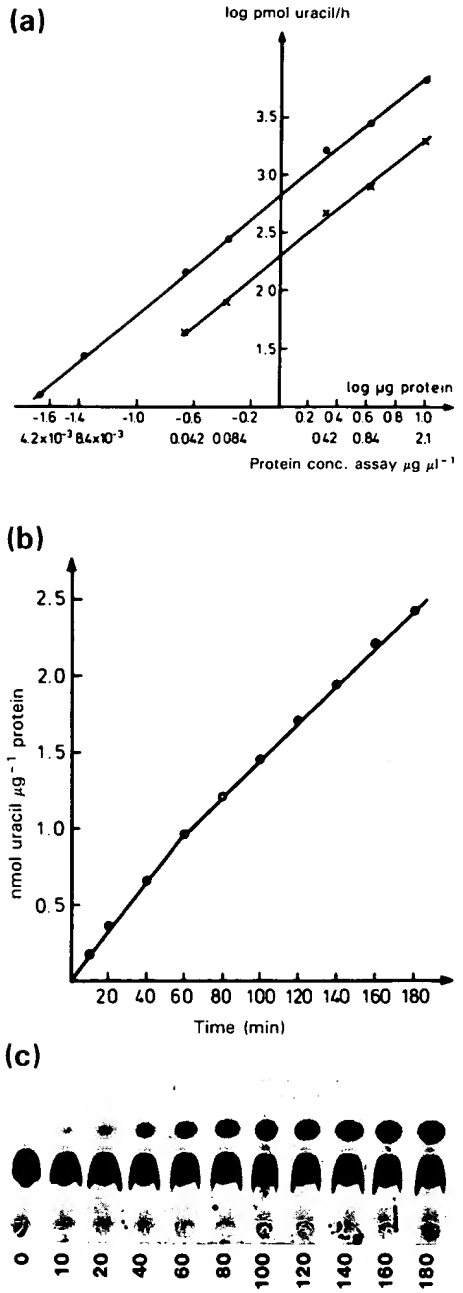

Figure 6. Cytosine deaminase activity from the 35S*codA* 3′*nos* reporter gene in *L. japonicus*.
(a) Double logarithmic plot of cytosine deaminase activity as a function of protein content. (•) Standard assay. (×) Assay with 10 mM cytosine. The actual protein concentrations in µg µl^{-1} are also given. (b) Cytosine deaminase activity measured by the standard assay as a function of time. (c) Autoradiogram of a time course experiment used for (b), protein concentration 0.06 µg ml^{-1}.

synthesis and the appearance of a number of fluorinated pyrimidine compounds after administration of 5-FU was previously described for mammals (Harbers *et al.*, 1959). Support for the primary 5-fluoro-dUMP inhibition of plant thymidylate synthase activity came from growth of tobacco on the precursor 5-fluoroorotate (5-FOA). The strong

inhibitory effect of 5-FOA on shoot growth was reversed by thymidine alone (data not shown). To explain the above observations, the ineffective cytosine reversion, and the uracil enhancement of the 5-FC effect, additional knowledge of pyrimidine uptake, translocation, enzyme affinities and metabolic control mechanisms of the pyrimidine pathways is needed.

The positive selection is based on simultaneous inhibition of *de novo* synthesis and provision of an alternative biosynthetic route. Unfortunately the conversion of cytosine into UMP appears to be to slow in counteracting the effect of PALA completely. Results from pea (Bressan *et al.*, 1978) and *Vinca* (Kanamori *et al.*, 1980) suggest that conversion of uracil into uridine or UMP could be the rate-limiting step. Constitutive expression of a gene encoding uracil phosphoribosyltransferase together with the cytosine deaminase might therefore improve the positive selection, although pyrimidine uptake may also be limiting. Some additional investigation of alternative inhibitors and the selective conditions used might also provide more distinct phenotypes. Improvements are clearly necessary for species with larger seeds like *Lotus*, where positive selection under conditions used for tobacco is not possible.

The simple enzymatic assay and the independence of growth conditions used for plant cell culture or plant growth, makes the *codA* selection an attractive alternative to the negative selections based on indole-3-acetamide hydrolase (Depicker *et al.*, 1988) or nitrate reductase (Nussaume *et al.*, 1991). Certainly, lack of cytosine deaminase activity in barley, sugar beet, rape seed, soybean, pea and *Arabidopsis* (data not shown) suggest a wide applicability of the *codA* marker and reporter gene.

Experimental procedures

Nucleic acid manipulations

Standard methods described in Sambrook *et al.* (1989) were used for DNA and RNA manipulations. DNA-modifying enzymes were used according to the manufacturers instructions. The procedure of Dellaporta *et al.* (1983) with additional phenol, phenol/chloroform treatments, was used for extraction of DNA from plant tissue. Nucleic acids for hybridization were immobilized on Gene Screen, NEN DuPont. For Southern analyses conditions using dextran sulphate and formamide were used as outlined in method III of the Gene Screen instruction manual, except that 67°C and 0.015 M NaCl were used in the final wash. Northern analyses were according to the Gene Screen manual. Total RNA was extracted from 2–4 g of leaf material homogenized under liquid nitrogen and transferred frozen to a polypropylene centrifuge tube. To this 7.5 ml of 100 mM Tris pH 8.0, 50 mM EDTA, 500 mM NaCl, 5% mercaptoethanol, 4% Sarkosyl and 0.15 g ml^{-1} of CsCl were added. Extracts were shaken, incubated at 65°C for 15 min before being centrifuged at 10 000 r.p.m., 4°C, for 20 min. The supernatant was loaded on a 1.0 ml 5.7 M CsCl 100 mM EDTA cushion in SW 50 polyallomer tubes, and centrifuged at 160 000 × ***g*** for 17 h. Liquid above the cushion was removed and the tube above the cushion washed twice with water before removal of the cushion. Pelleted RNA was resuspended in 1.5 ml 10 mM Tris pH 7.5 and precipitated with 2.5 volume ethanol at −20°C for 30 min. After centrifugation at 10 000 r.p.m. for 20 min, RNA was dissolved in 1.5 ml 10 mM Tris, pH 7.5, 100 mM NaCl, and reprecipitated. RNA was finally dissolved in H_2O.

Construction of the codA *marker*

The *codA* coding sequence from position 1642 to the stop codon at position 2923 (Danielsen *et al.*, 1992) was PCR amplified using: (i) an N-terminal primer, 5'GAATCGATGTCGAATAACGCTTTACA AAC, changing the *E. coli* GTG translational start codon to ATG and introducing a *Cla*I site adjacent to the ATG; (ii) a C-terminal primer, 5'GATGATCAACGTTTGTAGTCGATGGCTTC, introducing a *Bcl*I site overlapping the TGA stop codon and eliminating the *Cla*I site with a point mutation at position 2913. Since the internal *Cla*I site is Dam methylated the *codA* coding sequence could then be subcloned as a *Cla*I/*Bcl*I fragment. The 35S promoter used in pNE7 extends from +4 to −1296, the 5'untranslated TMV Ω leader used was described by Scofield *et al.* (1992).

Plant growth

The *Lotus japonicus* 'Gifu' B-129 line was propagated and grown as described in Handberg and Stougaard (1992). The same conditions and nutrients were used for the tobacco plants. A medium consisting of half strength B5, without sucrose, and 0.4% gelrite was used for testing germination and growth. Pyrimidine bases, analogues, and orotate were dissolved at 10 mg ml^{-1} in 10 mM MES, pH 5.5. For negative selection a concentration of 5-FC at 250 μg ml^{-1} was used in standard experiments. Nucleosides and deoxynucleosides were dissolved at 100 mg ml^{-1} in 100 mM MES, pH 5.5. PALA was dissolved at 10 mg ml^{-1} in 30 mM MES, pH 5.5, 5-FOA at 10 mg ml^{-1} was adjusted to pH 6.0 with 1 M NaOH. When necessary, solutions were gently heated. All solutions were filter sterilized. Plate concentrations were: thymidine, cytidine, uridine, 500 μg ml^{-1}; deoxycytidine, 200 μg ml^{-1}; uracil, 500 and 100 μg ml^{-1}; cytosine, 500 and 50 μg ml^{-1}. Minimum inhibitory concentrations of 5-FC were: *Lotus*, 150 μg ml^{-1}; tobacco, 100 μg ml^{-1}.

Tissue culture

Transformation, regeneration and *in vitro* culture of *L. japonicus* was performed according to Handberg and Stougaard (1992). The leaf dish transformation procedure of Horsch *et al.* (1985) was used for tobacco. For negative selection 5-FC at 500 μg ml^{-1} was used as standard, the minimum inhibitory concentration was 400 μg ml^{-1}. Cytosine was used at concentrations up to 2 mg ml^{-1}, uracil up to 100 μg ml^{-1}.

Cytosine deaminase assay

Plant material was homogenized in 10-fold excess (v/w) of extraction buffer: 200 mM Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 2 mM PMSF. The standard assay was set up with 1 μCi 2-^{14}C cytosine in 10 μl of assay buffer, started by addition of 10 μl plant extract and then incubated at 37°C for 1 h. The assay buffer contained: 200 mM Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA; 2 mM PMSF. For assays with excess cytosine the assay buffer contained additionally 10 mM cytosine. After 1 h a 5 μl or 10 μl assay mix was spotted on to a DC-Alufolien cellulose Merck 5552 TLC plate and dried using a blow dryer. The

TLC plate was developed in *n*-butanol/H_2O 86/14 v/v for 7 h. The TLC was then blow dried, covered with Saran wrap and autoradiographed. For quantitation of cytosine deaminase activity the spot containing 2-^{14}C uracil was counted in a scintillation counter. Protein in extracts was determined according to Spector (1978).

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Claim 60
T-DNA gene 2

Plant Cell Reports

A negative selection scheme for tobacco protoplast-derived cells expressing the T-DNA gene *2*

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ABSTRACT

The amido hydrolase encoded by the T-DNA gene 2 catalyzes the conversion of indole-acetamide, α-naphthalene acetamide, and other substrate analogues into the corresponding auxins. As a result, only gene 2-expressing protoplast-derived tobacco cells can grow in medium containing low concentrations (0.2–1 μM) of α-naphthalene acetamide as auxin precursor. However, in a mixture of SR1 and SR1,gene 2$^+$ protoplast-derived cells, cross-feeding occurs and consequently no positive selection for gene 2 is obtained. A 100-times higher concentration of α-naphthalene acetamide (between 30 and 300 μM) provides a negative selection scheme. Only the tobacco cells expressing gene 2 are sensitive to the high naphthalene acetamide concentration and cannot grow to colonies, while cells lacking the gene 2 product regenerate calli even in mixed gene 2$^+$ and gene 2$^-$ cell populations. Thus, gene 2 might provide a unique biochemically defined marker to investigate mutations and gene inactivation.

INTRODUCTION

There are several pathways by which plants can convert tryptophan to the naturally occurring auxin indole-3-acetic acid (for a review, see Marumo, 1986). However, in micro-organisms such as Pseudomonas savastanoi and in plants transformed with the T-DNA of Agrobacterium tumefaciens, another pathway has been found, in which tryptophan is converted into indole-3-acetamide (IAM) and then to indole-3-acetic acid (IAA) . This pathway is believed to be foreign to plants since IAM has been found only in conjunction with high concentrations of IAA in plant seedlings and citrus fruits.

Oncogenic T-DNAs encode enzymes that produce auxin and cytokinin so that infected tissues can proliferate without the need of exogenous hormones (for a review, see Inzé et al., 1987). Gene 4 codes for an isopentenyl transferase involved in the production of the cytokinin isopentenyl adenosine. An amidohydrolase encoded by gene 2 hydrolyzes the intermediate indole-3-acetamide (IAM), formed by the product of gene 1, into indole-3-acetic acid (IAA) (Thomashow et al., 1986; Van Onckelen et al., 1986).

Because the gene 2 product also catalyzes the conversion of α-naphthalene acetamide (NAM) into the biologically active auxin α-naphthalene acetic acid (NAA) (Inzé et al., 1984), it was postulated that plant cells containing the amidohydrolase gene 2 product should be able to grow on NAM as the only auxin supplement. On the other hand, high concentrations of NAM would not be toxic for normal plant cells but could be deleterious for cells capable of converting NAM to NAA. On this basis, it has been shown that NAM in the medium allows negative selection of gene 2-expressing seedlings (Budar et al., 1986). Also, it has been demonstrated that gene 2-expressing petunia callus is strongly inhibited or killed while wild-type callus grows normally in the presence of 30 μM NAM (Klee et al., 1987).

The auxin requirements and auxin cytotoxicities for tobacco protoplasts and protoplast-derived cells have been studied in detail (Caboche, 1980; Caboche et al., 1984). Exogeneously applied NAA efficiently stimulates mitotic activity and growth of protoplast-derived cells only in the concentration range between 1 μM and 20 μM at high cell density and in the concentration range between 0.05 μM and 1 μM at low cell density. Based on these observations, we postulated that different NAM concentrations would be differentially growth-promoting or toxic depending upon whether cells were derived from the tobacco plant SR1 or a transgenic SR1 plant transformed with gene 2 (Budar et al., 1986). The results demonstrate that SR1 protoplast-derived cells only initiate cell division and grow to colonies when the medium is supplemented with 5 up to 250 μM NAM. On the other hand, gene 2-expressing protoplast-derived cells can be stimumated to grow by concentrations between 0.5 and 10 μM NAM but are sensitive to concentrations higher than 30 μM. These results allowed the development of a negative selection scheme for gene 2 expression which, together with the availability of the gene 2 probe, might become especially valuable for fundamental studies on gene inactivation, mutagenesis, and T-DNA stability.

MATERIALS AND METHODS

Plant Material

All studies were performed on Nicotiana tabacum cv. Petit Havana SR1 and on the transformed SR1 plant 2441-7 (Budar et al., 1986). Plant 2441-7 was obtained after leaf disc transformation by Agrobacterium C58C1Rif^R(pGV2441), selection for kanamycin resistance, and screening for the expression of gene 2. Genetic analysis showed the presence of only one functional gene 2 locus (Budar et al., 1986).

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Protoplast Regeneration and Tissue Culture

Protoplasts from sterile-grown SR1 or 2441-7 plants were isolated in K3 medium deprived of hormones and regenerated as described (Depicker et al., 1985). After protoplast isolation and two successive washings, the protoplasts were diluted to a final concentration of 10^5 protoplasts/ml. At that moment, the cytokinin BAP was added and the auxin NAA, normally added up to a concentration of 0.1 mg/l was omitted or substituted by different concentrations of NAM (Sigma). After 3 days an equal volume of medium was added and after 7 days the protoplast-derived cells were embedded in agarose discs (Shillito et al., 1983), which gives an average plating density of 2×10^4 cells/ml. The surrounding liquid medium was refreshed every week while gradually lowering the sucrose concentration by 0.1 M. Cell division was followed under the microscope and colony formation was scored 5 weeks after protoplast isolation.

Microcalli or leaf fragments were grown on Murashige and Skoog medium containing 0.7% agar supplemented with different concentrations of BAP, NAA, or NAM as indicated (Results).

RESULTS

Physiological Concentrations of NAM as Auxin for Tobacco SR1 Protoplast-derived Cells

For the regeneration of tobacco protoplasts, NAA is commonly used as the required auxin in the medium. Physiological concentrations of NAA lie between 1 and 20 µM (0.1 and 3 mg/l); lower concentrations fail to induce mitotic activity while higher concentrations are toxic for sustained growth and colony formation (Caboche et al., 1984). First, we determined whether NAM could similarly promote growth of protoplast-derived cells and thus substitute for NAA in the medium of regenerating SR1 tobacco protoplasts. Freshly isolated protoplasts prepared from SR1 were resuspended in K3 medium containing 1.3 µM (0.3 mg/l) BAP and 0–0.05–0.1–0.25–0.5–1–2–5–10–20–50 mg/l NAM, respectively (Figure 1).

In all media tested, SR1 protoplasts regenerated cell walls in 48 h. These protoplast-derived cells enlarged but did not divide in medium without NAA or containing up to 2 µM (0.25 mg/l) NAM, while SR1 cells in medium containing 3 to 250 µM (0.5 mg/l up to 50 mg/l) NAM showed mitotic activity and colony growth (Figure 1, triangles; Figure 2A). The speed with which divisions were apparent was related to the supplemented concentration of NAM. In 5 µM NAM it takes 2 weeks before mitotic activity is visible, while on 25, 50, and 250 µM NAM, cell divisions are apparent the third or fourth day after protoplast isolation. The retardation of growth on low NAM concentrations disappears however during the fourth week of colony formation and large greenish calli are formed, which sometimes initiate shoot formation. This difference in callus phenotype at low NAM concentrations is probably related to qualitative differences in the auxin effects of NAM compared to NAA. Concentrations of NAM between 25 and 250 µM yield high plating efficiencies with white, compact calli. This led to the conclusions that NAM can promote growth in auxin-free medium, and that unlike NAA, it is not toxic for plant cells in any tested concentration.

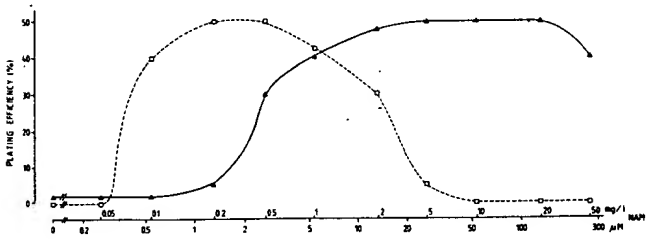

Figure 1. Plating efficiency of protoplast-derived cells of tobacco SR1 (▲) and SR1,2441-expressing gene 2 (□) as a function of different NAM concentrations. Protoplasts were isolated in the absence of hormones and plated at a density of 10^4 cells/ml in K3 medium supplemented with BAP (1.3 µM) and various concentrations of NAM. The plating efficiency was measured 5 weeks after protoplast isolation by scoring the number of colonies in respect to the initial number of cells. Different growth rates were not taken into account because this work aimed at the development of a negative selection scheme. The scale on top of the abscis line indicates the NAM concentrations in mg/l, while the subdivisions under the abscissa give the molar NAM concentrations.

Growth-promoting Activity of Different NAM Concentrations for Tobacco Protoplast-derived Cells Expressing Gene 2

After determining the concentration range in which NAM allows the regeneration of SR1 tobacco protoplasts to microcalli, we asked whether the conversion of NAM into NAA by the amidohydrolase in gene 2-transformed cells would alter the concentration range promoting their regeneration into calli. SR1,gene 2-transformed protoplast cells should be able to convert NAM to the more potent auxin NAA so that the first cell divisions can be induced by lower concentrations of NAM than for control SR1 cells. Concomitantly, the conversion of high concentrations of NAM into NAA might result in an inhibitory or toxic NAA concentration for gene 2-expressing protoplast cells, while untransformed cells could continue to proliferate. In order to test this possible clonal stimulation or inhibition of growth for cells expressing gene 2, protoplasts prepared from a plant transformed with gene 2 (SR1 2441-7; Budar et al., 1986) were washed and incubated in media supplemented with different concentrations of NAM.

The results are summarized in Figure 1 (squares) and shown in Figure 2B. Amidohydrolase expressing protoplast-derived cells (gene 2^+) regenerate efficiently into white microcalli in NAM concentrations between 0.5 and 10 µM. A NAM concentration of 25 µM still allows the initiation of the first cell divisions but strongly inhibits colony formation by stopping growth at a size of 5 to 20 cells. NAM concentrations of 50 to 250 µM block the first cell divisions of protoplast-derived cells and consequently do not allow colony formation. This sensitivity for high NAM concentrations of gene 2-transformed tobacco protoplast cells resembles the sensitivity of normal tobacco protoplast cells for high NAA concentrations (Caboche et al., 1984; Muller et al., 1985).

High NAM Concentrations Specifically Counterselect Gene 2-Expressing Protoplast-derived Cells

The results, summarized in Figure 1, clearly suggest that low and high NAM concentrations would promote differential growth of gene 2^- versus gene 2^+ protoplast-derived cells. In order to test whether a low and high NAM concentration could be used to select for or against gene 2-expressing cells in a mixed population, reconstruction experiments were set up. Non-transformed SR1 and gene 2^+-transformed protoplasts were mixed in different ratios (100/1, 10/1, 1/1, 1/10, and 1/100) after isolation and allowed to regenerate.

In low NAM concentrations (0.5 µM), both gene 2^- and gene 2^+ protoplast-derived cells grew and, unex-

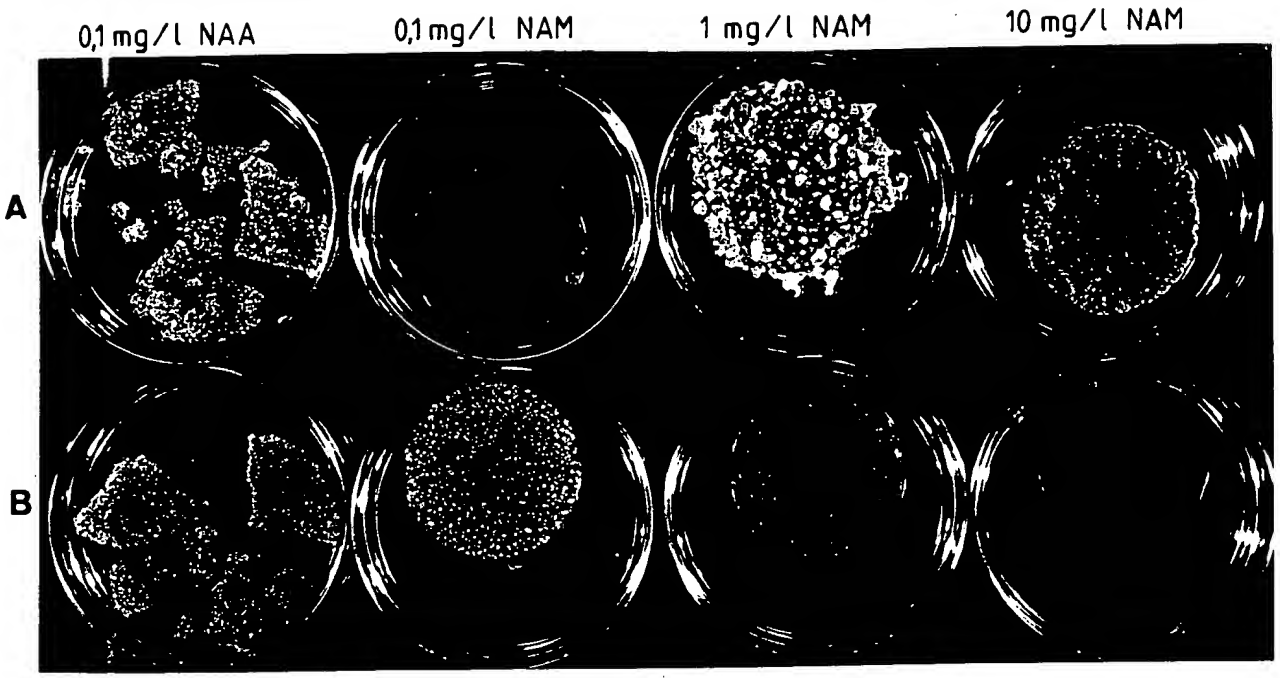

Figure 2. Differential growth-promoting activity of NAM on normal SR1 (A, top row) and on gene 2-expressing SR1,2441 (B, bottom row) protoplast-derived cells. The colony formation of both type of cells under normal hormone conditions is shown on the left. If 0.5 µM (0.1 mg/l) NAM is supplied, only gene 2-expressing cells can grow (positive selection). On the other hand, 50 µM (10 mg/l) NAM in the medium causes cell death only for gene 2-expressing cells (negative selection).

pectedly, no enrichment was obtained for gene 2^+ colonies. This can be explained by assuming that NAM is converted into NAA by the gene 2^+ cells and then released into the medium. Probably, cross-feeding of NAA (or another NAM metabolite) occurs so that positive selection for gene 2^+ cells in a mixture with gene 2^- cells is not possible. Therefore, the gene 2 marker cannot be used as an alternative to the antibiotic resistance gene markers.

On the other hand, in high NAM concentrations (> 50 µM), only SR1 protoplast-derived cells gave good plating efficiencies while the growth of SR1,gene 2^+ cells was efficiently counterselected. This means that the conversion of high concentrations of NAM into NAA is only toxic for the cells converting the NAM and not for the SR1 cells in the mixture. However, some escapes of gene 2^+ cells forming colonies were noticed especially in the reconstruction mixtures with few gene 2^+ cells (Breyne et al., in preparation). This type of background is presumably unimportant so long as the major use of this negative selection scheme is to select for gene 2^+ mutant cells in populations of gene 2^- cells.

Analysis of Gene 2 Inactivation in Regenerating leaf protoplasts

In order to determine the efficiency of the NAM counterselection for the expression of gene 2, we measured whether plant cells would escape the selection. Approximately 10^7 protoplasts were prepared from SR1,2441-7 leaves and incubated in medium supplemented with 50 µM NAM. Only one colony was obtained indicating a stringent counter selection for gene 2-expressing cells. At the moment, we are determining whether this colony is an escape or a mutant modified at the gene 2 locus or elsewhere.

In contrast to our observations on single cells, we noted that when the regenerating protoplasts were first grown to a critical size (approximately 50 cells) in medium containing 5 µM NAA, addition of up to 250 µM NAM could not stop colony formation. Thus, high auxin concentrations block the first divisions of protoplast cells but cannot block cell division in microcalli (> 20 cells). This parallels the selection conditions to obtain auxin resistance (Muller et al., 1985) : sensitivity to high auxin concentrations is only apparent when applied to protoplast-derived cells.

Morphology of Callus and Leaf explant Growth on Different Hormone Concentrations

After we established the conditions for positive and negative selection for gene 2 expressing tobacco cells, we determined whether they also were applicable on callus tissue. SR1 and SR1,2441-7 microcalli, regenerated from protoplasts and approximately 6 weeks old, were transferred onto solidified LS medium containing 1.3 µM BAP, and 0.5, 5, and 50 µM NAM, respectively. SR1 calli on the two lowest concentrations of NAM are green, form shoots, and can be distinguished from SR1,2441-7 calli which remain amorphic, compact, and white. On medium supplemented with 50 µM NAM both SR1 or SR1,gene 2-expressing calli grow as a very watery mass, although SR1,gene 2^+ calli are more inhibited. The cut edges of leaf explants react similarly to microcalli on this medium : gene 2-expressing plants develop white amorphous callus, while normal tobacco plants induce green callus and shoots.

When BAP is omitted from the medium and NAM is added in a concentration of 0.5 to 5 µM, the cut edges of SR1 leaves remain unresponsive though many branched roots can develop. However, the cut edges of leaves expressing gene 2 react with the growth of a rim of callus consisting of very large cells. The roots which develop from the leaf discs are thick, short, and not branched. Moreover, many are covered with root hairs and not positively geotropic.

These results show that in contrast to gene 2$^+$ protoplast-derived cells, gene 2-expressing calli cannot be positively or negatively selected, but calli and leaves with or without gene 2 can be distinguished morphologically by growth on different concentrations of NAM.

DISCUSSION

Tobacco protoplast cells do not show cell division in auxin-free medium, but the cells increase in size and remain metabolically active for at least a week. Increasing NAA concentrations between 0.5 and 15 µM can be correlated with an increasing number of dividing SR1 cells during the first week; however, this retardation of cell division disappears after 3 weeks and high plating efficiencies can be obtained at low auxin concentrations. High NAA concentrations (above 30 µM) inhibit colony formation from protoplast-derived cells or small cell clumps, especially at low cell density, but not when the microcalli obtain a critical cell size (more than 20 cells) (Caboche et al., 1984; Muller et al, 1985). Apparently cell clumps obtain a degree of autonomy in the regulation of the intracellular auxin concentration. The basis of auxin toxicity is not yet clear. NAA conjugates can be formed, presumably as detoxification by a specifically induced enzymatic pathway and it has been suggested that the auxin sensitivity is related to this NAA conjugation processes at low cell density (Caboche et al., 1984). These NAA conjugates are stored and although the enzyme activity is as yet not demonstrated, there is no doubt about the hydrolyzation of auxin amide conjugates. Our results show that NAM can stimulate cell divisions and promote growth of protoplasts-derived cells. NAM is taken up by tobacco protoplasts and exerts auxin effects, presumably after modification at a controlled rate. SR1 protoplast cells require ten times more NAM than NAA to initiate the mitotic activity of mesophyll protoplast-derived cells. However, while 25 µM NAA inhibited colony formation of SR1 cells, no upper inhibitory limit for NAM was found. SR1 cells expressing the amidohydrolase encoded by gene 2 showed the same dose-response curve for NAA as for NAM. Therefore, the observation that NAM at concentrations higher than 30 µM blocks cell division can be explained by the unregulated conversion of NAM to NAA in gene 2-expressing cells.

A clearly different physiological response dependent on the presence of the gene 2 product is seen with leaf explants on media supplemented with 1.3 µM BAP and 5 µM NAM : SR1 wounded leaves grow green shoot-forming callus while cut edges of SR1 gene 2-expressing leaves primarily grow compact, white callus. On medium with 5 µM NAM and without cytokinin, callus growth is stimulated primarily on the edges of gene 2-expressing leaf discs and abnormal, thick, and non-branched roots lacking positive geotropism are induced; in contrast, wild-type SR1 leaf discs remain unresponsive or produce normal roots into the medium.

Budar et al. (1986) described the screening of gene 2-expressing transgenic plants and demonstrated a negative selection scheme for gene 2-expressing seedlings. Here we report selection schemes applied to a large population of protoplast-derived cells. The sensitivity for high auxin concentrations is primarily apparent during the first cell divisions of protoplast-derived cells and during root development. Indeed, the counter selection of gene 2 on media with a high NAM concentration is similar to the selection conditions used to isolate auxin-resistant tobacco cells (Muller et al., 1985).

This method to counterselect tobacco cells expressing gene 2 may be very useful since most of the markers used for forward mutagenesis suffer from the disadvantage that the molecular basis is as yet unknown. The gene 2$^+$ → gene 2$^-$ selection scheme based on growth in medium with 50 µM NAM is stringent and allows subsequent analysis of the forward mutations at the DNA level by cloning the mutated locus. It is possible to score directly for gene 2 mutation frequencies in vivo or in vitro, either at spontaneous rates or induced by somaclonal variation in tissue culture or by mutagens (Breyne et al., in preparation). In addition, specific constructions can be designed to select for deletions or chromosomal rearrangements and for transposon insertions (De Greve et al., in preparation). Therefore, the selection scheme against gene 2 expression might become the counterpart of the selection schemes for HPRT mammalian cells (Stout and Caskey, 1985).

In summary, the gene-2-based reversible positive and negative growth conditions might become very valuable to assess gene activation versus inactivation, to determine gene mutation frequencies, and to probe for T-DNA stability in somatic and meiotic cells.

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The Anti-*nptII* Gene[1]

A Potential Negative Selectable Marker for Plants

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An efficient negative selection procedure is crucial to the isolation of rare homologous recombinants in gene targeting. Although gene targeting is a common practice in lower eukaryotes and is becoming routine in mammals, its application to plants has not been achieved. In this report, we have evaluated an antisense construct against the neomycin phosphotransferase gene (*nptII*) as a negative selectable marker. The anti-*nptII* gene construct was able to suppress *nptII* expression both transiently and in transformed tobacco (*Nicotiana tabacum*) calli. A construct was made which includes both a hygromycin-resistance gene and the sense plus antisense genes for neomycin phosphotransferase. Hygromycin-resistant calli were obtained after *Agrobacterium*-mediated transformation. Subsequently, hygromycin-resistant calli were tested for kanamycin sensitivity. The growth on kanamycin medium of calli harboring both the sense and antisense gene constructs was retarded, whereas that of control calli transformed with only the sense *nptII* gene was not inhibited. Southern blot analysis confirmed the presence of both *nptII* and anti-*nptII* genes. Northern blot analyses revealed that antisense transcripts of the *nptII* gene were made and that the level of sense transcripts was greatly reduced in transgenic calli. These results suggest that the anti-*nptII* gene could potentially be used as a negative selectable marker for gene targeting in plants.

Gene-targeting experiments in higher plants (Paszkowski et al., 1988; Baur et al., 1990; Lee et al., 1990; Offringa et al., 1990; Peterhans et al., 1990; Lyznik et al., 1991; Halfter et al., 1992) are currently restricted to positive selectable marker genes. These systems are not easily applicable to nonselectable traits. Because the frequency of homologous recombination events is low, an appropriate method has to be designed to discriminate against nonhomologous recombinants. Positive-negative selection strategies using the HSV-tk gene (Capecchi, 1989) and the activation of an introduced promoterless gene (Jeannotte et al., 1991; Bradley et al., 1992) have been successfully applied to murine embryo stem cells. It was the use of negative selection that made gene targeting so fruitful in mammalian systems (Thomas et al., 1986; Thomas and Capecchi, 1987; Chisaka and Capecchi, 1991; Hasty et al., 1991; Mombaerts et al., 1991; Chisaka et al., 1992; Donehower et al., 1992). Unfortunately, there are no universally reliable negative selectable markers available for higher plants. This is one of the major barriers for the application of gene targeting in higher plants. Without negative selection, a large population of transformants must be generated and maintained for screening rare homologous recombinants.

The HSV-tk gene was also our first choice as a negative selectable marker for higher plants. The coding sequence of the HSV-tk gene was inserted into an expression vector under the control of the CaMV 35S promoter. Subsequently, it was transformed into tobacco (*Nicotiana tabacum*) leaf discs and *Arabidopsis* roots via *Agrobacterium*-mediated gene transfer. The regenerated kanamycin-resistant plants were tested for gancyclovir sensitivity, which is encoded by HSV-tk. This negative selection did not function in our system as efficiently as it does in embryo stem cells. Transformed tissue showed obvious signs of gancyclovir toxicity, but during prolonged selection it was slow to develop callus (C. Xiang, L. Marton, and D.J. Guerra, unpublished observations). We did not investigate the possible causes, which include transcript instability, codon usage, uptake of gancyclovir, detoxification of gancyclovir by tobacco cells, etc. Rather, we sought to develop another method for negative selection in plants using antisense RNA.

Antisense RNA can be used to mimic mutations in both prokaryotic and eukaryotic organisms (Van der Krol et al., 1987; Takayama and Inouye, 1991). Although antisense RNA was originally found as a naturally occurring mechanism to control gene expression in bacteria (Tomizama et al., 1981; Mizuno et al., 1984), it was proposed that it could be used to inhibit eukaryotic gene expression (Izant and Weintraub, 1984, 1985). The application of antisense RNA in plants has been successful in effectively inhibiting the activity of nopaline synthase (Rothstein et al., 1987; Sandler et al., 1988), chloramphenicol acetyltransferase (Delauney et al., 1986; Ecker and Davis, 1986), chalcone synthase (Van der Krol et al., 1988), polygalacturonase (Sheehy et al., 1988; Smith et al., 1988), and β-glucuronidase (Robert et al., 1989). More recently, antisense RNA was used to inhibit a granule-bound

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Abbreviations: CaMV, cauliflower mosaic virus; HSV-tk, herpes simplex virus thymidine kinase; MS, Murashige and Skoog; *nos*, nopaline synthase gene; *nptII*, neomycin phosphotransferase II; *rbcS*, ribulose bisphosphate carboxylase small subunit termination signal.

starch synthase (Visser et al., 1991), the synthesis of ethylene (Hamilton et al., 1990), another rate-limiting enzyme in the ethylene biosynthesis pathway, ACC synthase (Oeller et al., 1991), and the ADP-Glc pyrophosphorylase (Muller-Rober et al., 1992). Antisense RNA to *nptII* was successfully used in a mammalian system (To et al., 1986).

Antisense RNA methodology appropriately fits into the positive-negative selection system for gene targeting. We reasoned that an antisense gene could provide a negative selectable marker if it blocked sense marker gene expression. The positive-negative strategy is as follows: a positive selectable marker gene is placed within the genomic flanking sequences, and its antisense gene resides outside the homologous region. If random integration or a nonhomologous recombination event occurs after transformation, the antisense gene is incorporated into the genome and the transformant will be sensitive to the antibiotic because the antisense RNA will block marker gene expression. If the antisense gene is not incorporated and only the selectable antibiotic-resistance gene is integrated into the genome, directed by the homologous sequences, the transformant will be resistant to the antibiotic. In this way, a single selection should recover predominantly homologous recombination events. To evaluate this antisense RNA strategy as a means of negative selection for gene targeting in higher plants, we chose the *nptII* gene as a model system, since *nptII* is a reliable selectable marker widely used for plant transformation.

MATERIALS AND METHODS

Plasmid Construction

Four anti-*nptII* gene constructs were made for stable transformations using the transposon Tn5 coding sequence. To create pCX403 and pCX404 (Fig. 1), a 1000-bp *Bgl*II-*Sma*I and a 650-bp *Bgl*II-*Nae*I fragment of Tn5 including a 35-bp untranslated region and translation start codon were isolated from pGA342 (a gift from G. An, Washington State University) and inserted into an expression binary vector, pGA643 (An et al., 1988), at *Bgl*II and *Hpa*I sites so that Tn5 coding sequences were in the reverse orientation. The *Bgl*II-*Sma*I and *Bgl*II-*Nae*I fragments of pGA342 were inserted into pBluescript II KS$^-$ at *Bam*HI and *Eco*RV sites to create pCX401 and pCX402, respectively. The Tn5 coding sequences were then released as *Hin*dIII-*Xba*I fragments from pCX401 and pCX402 and inserted into the pKYLX7 (Schardl et al., 1987) expression cassette in the inverted orientation at corresponding restriction sites to form pCX407 and pCX408 (Fig. 1).

For the transient expression experiment, two plasmids, pBSIIKS$^-$/*npt*$^+$ and pBSIIKS$^-$/*npt*$^-$, containing the *nptII* or anti-*nptII* gene, respectively, were constructed in pBluescript II KS$^-$. The anti-*nptII* chimeric gene was isolated from pCX407 as an *Eco*RI/*Cla*I fragment and inserted into pBluescript II KS$^-$ at corresponding sites to give pBSIIKS$^-$/*npt*$^-$. To create pBSIIKS$^-$/*npt*$^+$, the *nptII* coding sequence was isolated as a 1.0-kb *kpn*I/*XBa*I (blunt-ended) fragment from pCX401 and inserted into pKYLX6 (Schardl et al., 1987) at *Kpn*I and *Hin*dIII (blunt-ended) sites, so that *nptII* was regulated by the CaMV 35S promoter and the terminator of the *rbcS* gene. The whole cassette, as an *Eco*RI/*Cla*I fragment, was inserted into pBluescript II KS$^-$ to form pBSIIKS$^-$/*npt*$^+$.

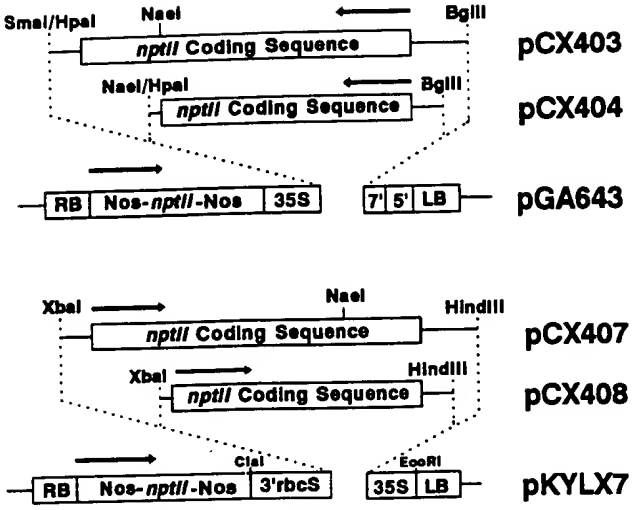

Figure 1. Schematic representation of the anti-*nptII* gene constructs for kanamycin-sensitivity assays. The plasmid construction was described in "Materials and Methods." 35S, CaMV 35S promoter; 3'rbcS, polyadenylation signal of the *rbcS* gene; RB and LB, right and left border of T-DNA; *nptII*, neomycin phosphotransferase gene; Nos-*nptII*-Nos, chimeric *nptII* gene regulated by the promoter and terminator of *nos*; 7' and 5', polyadenylation signal of gene 7 and gene 5 of the Ti plasmid. Arrows indicate the orientation of the *nptII* sequence.

The plasmid pCX407/hpt was constructed for the stable transformation of tobacco (*Nicotiana tabacum*) by modifying the plasmid pCX407 to contain a hygromycin-resistance gene. The hygromycin-resistance gene regulated by the CaMV 35S promoter and the polyadenylation signal of *nos* was isolated from the plasmid pGA883 (a gift from G. An, Washington State University) as an *Xba*I fragment. The *Xba*I fragment was blunt-ended by treating with T4 DNA polymerase (Promega) and was inserted into pCX407 at a *Cla*I site, which was blunt-ended in the same way. *Escherichia coli* strain DH10B was used for all cloning. All plasmid DNA manipulations were performed as described (Ausubel et al., 1987; Sambrook et al., 1989).

Transient Expression via Electroporation and NPTII Assay

Protoplasts were isolated from 4-d-old tobacco NT-1 suspension cells as described (Michael et al., 1988). Briefly, suspension cells were harvested and incubated in 0.5 M mannitol (pH 5.7) for 30 min. The plasmolyzed cells were then digested in an enzyme solution consisting of 0.5% Macerozyme R-10, 2.0% cellulase R-10, and 0.5 M mannitol, pH 5.7, at 30°C for a few hours with gentle shaking on an orbital shaker. Protoplasts were purified by filtering through 75-μm nylon mesh and washing three times in 0.5 M mannitol (pH 5.7) at 100*g*. Protoplasts were finally resuspended in 0.5 M mannitol (pH 5.7), adjusted to 5×10^5/mL, and stored on ice for subsequent electroporation. Electroporation was conducted as described (Saunders et al., 1989) using a square-wave pulse generator (Electro Cell Manipulator, model 600, BTX) and 2-mm gap electroporation chambers. The NPTII assay was performed as described (Platt and Yang, 1987).

Agrobacterium-Mediated Transformation

Plasmids for plant transformation were introduced into *Agrobacterium tumefaciens* LBA4404 or EHA105 via the freeze-and-thaw method (An et al., 1988). The intactness of the plasmid in agrobacteria was verified by plasmid miniprep and restriction analysis before transformation. *Agrobacterium*-mediated transformation of tobacco leaf discs was conducted as described (An et al., 1988). Briefly, growth chamber-grown. *N. tabacum* cv Xanthi leaves were surface-sterilized in 20% bleach (1.0% sodium hypochlorite). After three rinses with sterile distilled water, leaf discs were cut with a sterile cork borer. Cocultivation was carried out in liquid medium for 3 d in the dark. After cocultivation, leaf discs were transferred to callusing and shooting medium containing various concentrations of kanamycin sulfate for selection, and 500 mg/L of carbenicillin and 250 mg/L of cefotaxime to inhibit agrobacteria. For stable transformation experiments, leaf pieces were transferred to the MS callusing medium containing 50 mg/L of hygromycin or 300 mg/L of kanamycin (for pKYLX7 control only) for selection. All cultures were maintained under a 16-h photoperiod at 26°C. All tobacco plants were grown under a 16-h photoperiod at 26°C and 60% RH.

DNA and RNA Isolation

Total DNA was isolated from callus tissue by the cetyltrimethylammonium bromide method as described (Ausubel et al., 1987). Total callus RNA was isolated using a guanidinium isothiocyanate procedure (Chirgwin et al., 1979) with an RNA Isolation Kit (Promega). Nucleic acid concentrations were quantified spectrophotometrically.

Southern and Northern Blots

For Southern blotting, 10 μg of total DNA was cut, fractionated on a 0.7% agarose gel, and blotted onto a nitrocellulose membrane via capillary transfer. *Bst*EII-digested λ DNA was used as DNA size markers. A digoxigenin-labeled probe was prepared using a random primer-labeling and detection kit (Boehringer Mannheim). Hybridization was carried out in 5× SSC at 65°C in a rotary hybridization oven. The filter was washed twice at room temperature for 15 min each in 2× SSC with 0.1% SDS, then in 0.1× SSC with 0.1% SDS, and finally in 0.1× SSC with 0.1% SDS at 65°C for 15 min. For northern blot analysis, 10 μg of total RNA was fractionated on a formaldehyde agarose gel (1.0%) and transferred onto a nitrocellulose membrane. The manufacturer's instructions for hybridization and detection using the same nonisotopic kit were followed. The sense- and antisense-specific probes were generated via a modified asymmetric polymerase chain reaction method (McCabe, 1990) using one single primer and the linearized plasmid, pCX401, containing the *nptII* insert as the template. Briefly, the template plasmid pCX401 was linearized with either restriction endonuclease *Hin*dIII (for the antisense-specific probe) or *Kpn*I (for the sense-specific probe). The reaction mixture was assembled as standard for polymerase chain reaction (20 mM Tris-HCl, pH 8.3 at 20°C, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 20, 100 μg/mL of autoclaved gelatin, 50 μM dATP, dGTP, and dCTP, 32.5 μM dTTP, 2 units of *Taq* DNA polymerase) except that only one primer (20 pmol) and 17.5 μM digoxigenin-11-dUTP (Boehringer Mannheim) were included. The identical conditions, except for the primers, were used to generate both probes. The SK primer (Stratagene) was used for generating the sense-specific probe and the KS primer for the antisense-specific probe.

RESULTS AND DISCUSSION

Transient Expression Assay in Tobacco Protoplasts

The plasmids pBSIIKS$^-$/*npt*$^+$ containing the sense *nptII* gene, pBSIIKS$^-$/*npt*$^-$ containing the anti-*nptII* gene, and pBluescript II KS$^-$ as control were used for transient expression assays to assess whether the anti-*nptII* RNA was able to inhibit the expression of the *nptII* gene (see "Materials and Methods"). The transient expression assay results are shown in Figure 2. When plasmids pBSIIKS$^-$/*npt*$^+$ and pBSIIKS$^-$/*npt*$^-$ in equal molar amounts were coelectroporated into tobacco protoplasts, the NPTII activity was reduced (lane 3) as compared with the sample electroporated with plasmid pBSIIKS$^-$/*npt*$^+$ alone (lane 4). Lanes 1 and 2 were negative controls. This result suggested that the anti-*nptII* gene reduced *nptII* gene expression, presumably due to the antisense RNA effect. However, there is still a substantial amount of transient NPTII activity in protoplasts receiving both sense and antisense *nptII* genes. Whether the NPTII activity is low enough to allow negative selection in protoplasts has yet to be determined. This is critical for plant gene targeting, since direct gene transfers using a protoplast system provide a high frequency of transformation, which compensates for the low frequency of homologous recombination events. Further experiments should be conducted to demonstrate the negative selection in stably transformed protoplasts.

Kanamycin-Sensitivity Assay after *Agrobacterium*-Mediated Transformation

The four constructs pCX403, pCX404, pCX407, and pCX408 (Fig. 1), all containing both sense and antisense *nptII* genes, were used to transform tobacco leaf discs via *Agrobacterium*-mediated transformation. The plasmid pCX403, containing a full-length anti-*nptII* gene, and pCX404, containing

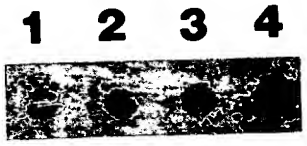

Figure 2. Transient expression assay of the anti-*nptII* gene constructs. Tobacco protoplasts (0.5 mL) in 0.5 M mannitol (pH 5.7) at 5×10^5/mL were mixed with 30 μL of a DNA solution containing 20 μg of supercoiled plasmid DNA and 50 μg of calf thymus DNA as carrier. Two days after electroporation, protoplasts were collected for NPTII activity assay. Electroporation and NPTII assay were described in "Materials and Methods." Lane 1, No plasmid; lane 2, 20 μg of pBluescript II KS$^-$; lane 3, 10 μg of pBSIIKS$^-$/*npt*$^+$ plus 10 μg of pBSIIKS$^-$/*npt*$^-$; lane 4, 10 μg of pBSIIKS$^-$/*npt*$^+$ plus 10 μg of pBluescript II KS$^-$.

two-thirds of the full-length gene, were designed to examine the effect of the length of the anti-*nptII* gene on *nptII* expression. The plasmids pCX407 and pCX408 were a duplication of pCX403 and pCX404 except that a different polyadenylation signal sequence was used. These plasmids and pGA643, which served here as a positive control for transformation, were introduced into *A. tumefaciens* strain EHA105, where their structures were verified by restriction analysis. The *A. tumefaciens* EHA105 harboring the anti-*nptII* gene constructs or pGA643 were used to transform tobacco leaf discs via the cocultivation method (An et al., 1988). After cocultivation, agrobacteria were washed off and leaf discs were cultured on MS callusing and shooting media with various kanamycin concentrations. The number of calli that emerged from each leaf disc were scored in about 4 weeks.

All four antisense constructs completely eliminated callus and shoot formation when transformed tobacco leaf discs were cultured on media containing 200 mg/L of kanamycin (Fig. 3, Table I). The same effect was observed at a higher kanamycin concentration. Concentrations lower than 200 mg/L did not completely inhibit callusing or shoot formation (Table I). This indicates that the kanamycin-resistant phenotype is overcome by antisense RNA if sufficient antibiotic is supplied to the selection media. Because the anti-*nptII* constructs used in our experiments prevented us from recovering stable transformants, it could be argued that these results might be due to an artifact of intramolecular homologous recombination, which could lead to inversion for pCX403 and pCX404 and deletion for pCX407 and pCX408. The possible inversion for pCX403 and pCX404 could invert the DNA sequence (Nos terminator and 35S promoter) between the inverted repeats of *nptII*, resulting in the antisense *nptII* flanked by two terminators and the sense *nptII* flanked by two promoters. Both inversion and deletion would yield a kanamycin-sensitive phenotype. Therefore, further confirmation of the antisense RNA effect was required and the following experiments were designed to address these issues.

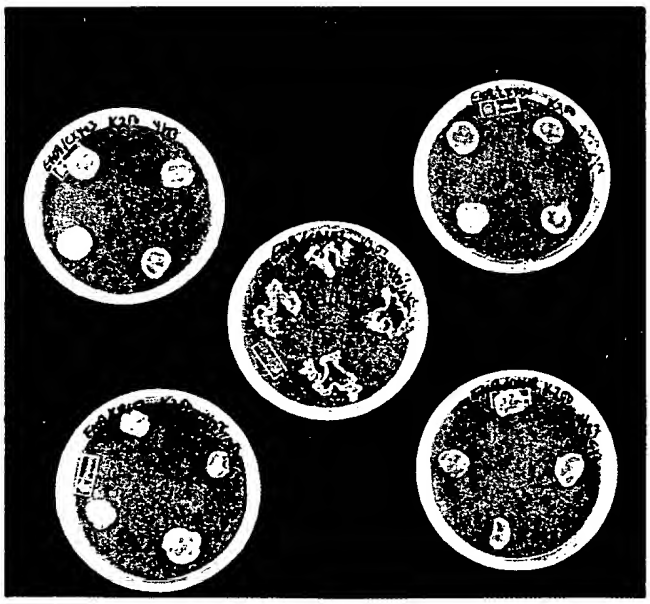

Figure 3. Selection of transformed tobacco leaf discs on 200 mg/L of kanamycin. The plasmid pGA643-transformed leaf discs (positive control) are in the center plate. Discs transformed with the four anti-*nptII* gene constructs (Fig. 1) are flanking the control Petri dish. No calli were generated in the tobacco leaf discs harboring the anti-*nptII* gene constructs.

Table I. *Number of calli emerged per leaf disc on MS media containing various kanamycin concentrations following transformation with different gene constructs*

+++, Calli formed all over the cutting edges (unable to count). For each transformation, five different kanamycin concentrations (0, 50, 100, 200, and 400 mg/L) were tested. For each kanamycin concentration, triplicate plates were used. Four leaf discs were placed on each plate. These plates were incubated at 25°C under a 16-h photoperiod. Values are the average of two experiments.

Construct	Kanamycin Concentration (mg/L)				
	0	50	100	200	400
pGA643	+++	+++	+++	+++	+++
pCX403	+++	4.50	2.45	0.00	0.00
pCX404	+++	4.50	2.45	0.00	0.00
pCX407	+++	4.51	2.55	0.00	0.00
pCX408	+++	4.20	2.15	0.00	0.00
Untransformed	+++	0.00	0.00	0.00	0.00

Anti-*nptII* Expression in Transformed Tobacco Calli

To investigate whether our kanamycin sensitivity assay results were the effect of antisense RNA, the plasmid pCX407/hpt (Fig. 4) was used to generate stable transformants for molecular analysis. This plasmid and pKYLX7 as control were introduced into *A. tumefaciens* strain LBA4404 and subsequently used to transform tobacco leaf discs as described (An et al., 1988). Hygromycin-resistant calli were generated. Individual hygromycin-resistant calli were maintained on the hygromycin-containing MS callusing medium. For the kanamycin sensitivity test, hygromycin-resistant calli were transferred to the MS callusing medium containing 200 mg/L of kanamycin. All seven hygromycin-resistant callus lines that were tested were inhibited on kanamycin-containing medium as judged by the lack of fresh weight increase of calli (data not shown), indicating that the anti-*nptII* genes are functional in transformed calli and are able to reduce NPTII activity, probably because of the antisense RNA effect. This suggested that anti-*nptII* constructs could be utilized for negative selection. To examine if it was the anti-*nptII* gene that caused the loss of, or decrease in, kanamycin resistance, four representative calli sensitive to kanamycin were chosen for further molecular analysis.

Transformation of the four calli chosen was confirmed by Southern blot analysis. Total DNA isolated from the transformed calli, as well as the control, was digested with restriction endonucleases *Eco*RI and *Bam*HI, fractionated on a 1.0% agarose gel, and transferred onto a nitrocellulose filter. As shown in Figure 4B, all kanamycin-sensitive calli (lanes 1–4) contained a DNA fragment of 2.3 kb and another fragment of 3.4 kb that hybridized to the *nptII* probe, indicating the integration of both the *nptII* and the anti-*nptII* genes. By

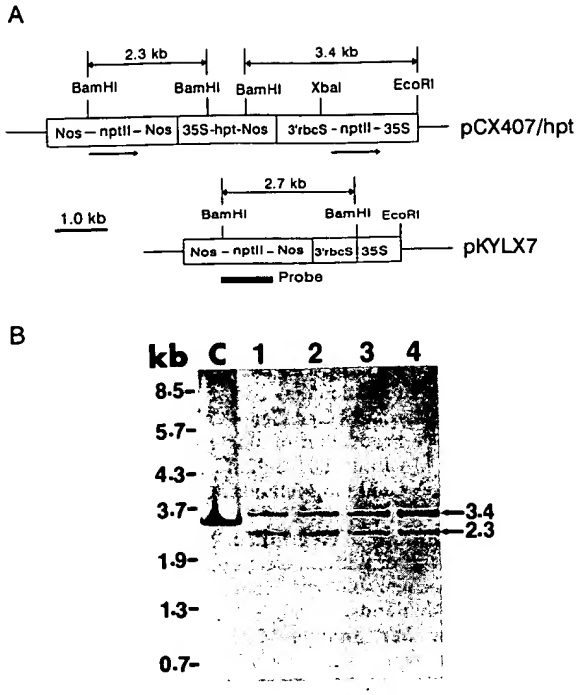

Figure 4. Southern blot analysis of the transformed calli. A, Schematic representation of pCX407/hpt and pKYLX7. 35S, CaMV 35S promoter; 3'rbcS, polyadenylation signal of the *rbcS* gene; Nos-*nptII*-Nos, *nptII* gene regulated by the promoter and terminator of *nos*; 35S-hpt-Nos, hygromycin-resistance gene regulated by the 35S promoter and the terminator of *nos*. Bold arrows indicate the orientation of the *nptII* sequence. B, Southern blot analysis of transformed calli. Total DNA was restricted with *Bam*HI and *Eco*RI, fractionated on a 1.0% agarose gel, blotted onto a nitrocellulose membrane, and probed with digoxigenin-labeled *nptII* gene sequence. *Bst*EII-digested λ DNA size markers are shown on the left. Lane C, Control. Lanes 1 to 4, Individual transformed calli.

contrast, the control callus (lane C) transformed with pKYLX7 vector alone contained only a DNA fragment of 2.7 kb, indicating the presence of the *nptII* gene. *Agrobacterium* contamination was ruled out, since no hybridization signal was detected when a duplicate filter was probed with a labeled pKYLX7 vector segment excluding the T-DNA region (data not shown). Moreover, intramolecular homologous recombination would lead to the deletion of the hygromycin-resistance gene due to the direct repeat sequences of *nptII*. This should result in a hygromycin-sensitive phenotype, which would not be recovered upon selection in this experiment. We do not know the frequency of this potential intramolecular homologous recombination in plant cells; presumably, such events are rare. Regardless of the frequency of such events, it should not interfere with the positive-negative selection strategy because intramolecular recombination, if it occurs at all, will lead to either excision (*nptII* and anti-*nptII* in the same orientation) or inversion (*nptII* and anti-*nptII* in the opposite orientations). Both excision and inversion events would disrupt the *nptII* gene, both resulting in the loss of kanamycin resistance, which is selected against in the positive-negative selection strategy of gene targeting.

Northern blot analysis was performed to estimate the expression of both sense and antisense *nptII* genes. Total RNA was isolated from the transformed calli as well as control, separated on formaldehyde agarose gels, and blotted onto nitrocellulose filters. Two duplicate filters were probed with either the sense or antisense probe. Both probes were labeled similarly. As shown in Figure 5, in control callus (lane C in A and B), only sense transcripts of *nptII* were detected at high level, whereas antisense transcripts were not detected. In calli sensitive to kanamycin (lanes 1–4 in A and B), both sense and antisense transcripts were detected, whereas the level of sense transcripts was greatly reduced compared with that in control callus. The level of antisense transcripts was high. It has been proposed that antisense RNA could function at either the translational level by inhibiting ribosome binding or at an earlier stage by forming double-stranded RNA complexes, which could be substrates for endogenous ribonucleases (Takayama and Inouye, 1990). The promoter strength might partially contribute to the reduced sense *nptII* transcripts because the *nos* promoter was used for the sense *nptII* expression and the 35S promoter for the antisense *nptII*. It is generally recognized that the 35S promoter is much stronger than the *nos* promoter (Sanders et al., 1987). The *nos* promoter was deliberately chosen because the use of a weaker promoter for the sense *nptII* should further enhance the antisense RNA effect when the anti-*nptII* is expressed under a stronger promoter such as the 35S.

In conclusion, our results demonstrate that *nptII* gene expression can be repressed by expressing its antisense gene. The kanamycin-resistance phenotype is overcome by antisense RNA if sufficient kanamycin is supplied to the selection medium. Therefore, the anti-*nptII* gene has potential as a negative selectable marker for gene targeting in higher plants. Other negative selection genes have been reported (Depiker et al., 1988; Hilson et al., 1990; Czako and An, 1991). The amido hydrolase gene 2 of *Agrobacterium* T-DNA was proposed for the elimination of a specific group of cells or tissues and for fundamental studies on gene inactivation (Depiker et al., 1988). A chimeric *RAS2* gene from yeast also had a "killer" effect in *N. plumbaginifolia* (Hilson et al., 1990). The expression of diphtheria toxin chain A gene in tobacco was demonstrated to be toxic (Czako and An, 1991). These negative

Figure 5. Northern blot analysis of transformed calli. Total RNA (10 μg) was fractionated on a formaldehyde agarose gel, blotted onto a nitrocellulose filter, and hybridized with the digoxigenin-labeled sense-specific (A) or antisense-specific (B) probe. Both probes were similarly labeled. A, Level of *nptII* transcripts. B, Level of anti-*nptII* transcripts. Lanes C, Control. Lanes 1 to 4, Individual transformed calli.

selection genes could also potentially be used in a positive-negative selection strategy for plant gene targeting. The anti-*nptII* system we reported here is a straightforward and relatively simple alternative to these negative selection genes. We are further developing the positive-negative strategy using anti-*nptII* as the negative selectable marker gene for specific delivery of homologous sequences to the plant genome but are aware of its potential application to other eukaryotic systems where antisense RNA has been shown to inhibit gene expression.

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